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Sleep architecture and homeostasis in mice with partial ablation of melanin-concentrating hormone neurons

Christophe Varin[a,b], Sébastien Arthaud[a,b], Denise Salvert[a,b], Nadine Gay[a,b], Paul-Antoine Libourel[a,b], Pierre-Hervé Luppi[a,b], Lucienne Léger[a,b], Patrice Fort[a,b,*]

[a] Neuroscience Research Center of Lyon (CRNL), CNRS UMR 5292, INSERM U1028, SLEEP Team, Lyon, France
[b] Université Claude Bernard, Lyon 1, Lyon, France

HIGHLIGHTS

- Using an original model of transgenic mice, we provide new insights regarding the contribution of the hypothalamic MCH-expressing neurons to the regulation of vigilance states.
- We indeed show that adult MCH^{Atax} mice with a severe alteration of central MCH system depicted subtle but deleterious qualitative and quantitative alterations of sleep.
- Whereas MCH neurons are thought to be involved in REM sleep physiology, our data suggests that when activated they also improve the depth of slow wave sleep (SWS) by favoring both its consolidation and related slow-wave activity (SWA).
- Such mechanisms could be recruited for restoring function of sleep especially following homeostatic challenge as sleep deprivation or learning/memory processes.

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ABSTRACT

Recent reports support a key role of tuberal hypothalamic neurons secreting melanin concentrating-hormone (MCH) in the promotion of Paradoxical Sleep (PS). Controversies remain concerning their concomitant involvement in Slow-Wave Sleep (SWS). We studied the effects of their selective loss achieved by an Ataxin 3-mediated ablation strategy to decipher the contribution of MCH neurons to SWS and/or PS. Polysomnographic recordings were performed on male adult transgenic mice expressing Ataxin-3 transgene within MCH neurons (MCH^{Atax}) and their wild-type littermates (MCH^{WT}) bred on two genetic backgrounds (FVB/N and C57BL/6). Compared to MCH^{WT} mice, MCH^{Atax} mice were characterized by a significant drop in MCH mRNAs (−70%), a partial loss of MCH-immunoreactive neurons (−30%) and a marked reduction in brain density of MCH-immunoreactive fibers. Under basal condition, such MCH^{Atax} mice exhibited higher PS amounts during the light period and a pronounced SWS fragmentation without any modification of SWS quantities. Moreover, SWS and PS rebounds following 4-h total sleep deprivation were quantitatively similar in MCH^{Atax} *vs.* MCH^{WT} mice. Additionally, MCH^{Atax} mice were unable to consolidate SWS and increase slow-wave activity (SWA) in response to this homeostatic challenge as observed in MCH^{WT} littermates. Here, we show that the partial loss of MCH neurons is sufficient to disturb the fine-tuning of sleep. Our data provided new insights into their contribution to subtle process managing SWS quality and its efficiency rather than SWS quantities, as evidenced by the deleterious impact on two powerful markers of sleep depth, *i.e.*, SWS consolidation/fragmentation and SWA intensity under basal condition and under high sleep pressure.

1. Introduction

Evidence implicates the tuberal hypothalamic area (THA) as an integrative center for the regulation of paradoxical sleep (PS), upstream to PS-promoting brainstem networks [1–4]. Recent data in rodents point to the involvement of THA neurons co-expressing melanin-concentrating hormone (MCH), CART, Nesfatin-1, GAD67 and vGluT2 in PS regulation [3,5–11]. These so-called MCH neurons send widespread efferents consistent with the brain distribution

* Corresponding author at: Centre de Recherche en Neurosciences de Lyon (CRNL), CNRS UMR5292—INSERM U1028, Faculté de Médecine Lyon–Est–Laënnec/La Buire—UCBL 1, 7, rue Guillaume Paradin, 69372 LYON Cedex 08, France. Fax: +33 4 78 77 10 22.
E-mail address: patrice.fort@univ-lyon1.fr (P. Fort).

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of MCH-R1 receptors and their potential contribution to SWS or PS [12–18]. They express the immediate early gene c-Fos after PS rebound following short (6 h) or long-lasting (72 h) PS deprivations in rats [10,11,19–21] and mice [22]. Their firing is quite specific to PS as revealed by juxtacellular recordings across natural sleep–waking cycle in head-restraint rats [23]. In mice, chronic optogenetic activation of MCH neurons at the beginning of the active period increases SWS and PS. The same protocol during the sleep period promotes PS and increases the probability of SWS to PS transition [24,25]. Furthermore, the acute optogenetic stimulation of MCH neurons during SWS increases the probability of PS occurrence whereas stimulation during PS extends the duration of the current episode [26]. However, no drastic effects were reported following their acute or chronic silencing achieved with inhibitory optogenetic tools [25,26]. The precise contribution of MCH neurons to SWS and/or PS regulation remains elusive despite an obvious experimental effort in recent years [27]. To bridge this gap, we took the opportunity to record sleep in transgenic mice with a targeted ablation of MCH cell bodies and their axons [28]. We used to attain this goal a toxin-mediated genetic cell ablation strategy consisting of a truncated Machado–Joseph disease gene product (Ataxin-3) under the control of an *MCH* gene promoter in FVB/N and C57BL/6 transgenic mice. Pro-apoptotic Ataxin-3 accumulates in MCH neurons from the first post-natal week, with a maximal loss of MCH cell bodies by post-natal week 15 [28].

Here, we found that adult MCH^{Atax} mice, endowed with a severe although partial impairment of MCH system, depicted subtle but deleterious qualitative and quantitative alterations of both SWS and PS during spontaneous sleep–wake cycle and recovery period following a total sleep deprivation. This suggests that the MCH neurons, when activated, improve SWS depth by favoring both its consolidation and related slow-wave activity (SWA). Such mechanisms could be recruited for restoring function of sleep especially following homeostatic challenge.

2. Material and methods

2.1. Characterization of MCH^{Atax} mice bred on C57BL/6 and FVB/N genetic backgrounds

2.1.1. Animal care

Experiments were carried out in strict accordance with the European Communities Council Directive (86/609/EEC) and recommendations in the guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH publication n° 85–23). Protocols were approved by the local "Comité d'Éthique en Expérimentation Animale de l'Université LYON 1 (C2EA)". Breeding was done within the local "ALECS conventionnel" facility. Offspring were genotyped (at post-natal day 10) by PCR amplification of genomic DNA using the following primer sets (Eurofins, France): forward, CGGGACCTATCAGGACAGAGT; reverse, TCGCCTGCGACGGCGGCATCT [28]. Once genotyped, mice were weaned at 3–4 weeks of age, grouped and housed according to gender. Only male transgenic (MCH^{Atax}) and wild-type (MCH^{WT}) littermates were used.

2.1.2. Breeding of FVB/N transgenic mice

Two 4-month old MCH^{Atax} males, provided by Pr. J.M. Friedman (The Rockefeller University, New York, USA), were the founders. The protocol for their generation has been previously detailed [28]. Our colony was maintained for the duration of experiments with a standard mating system: one MCH^{Atax} male with 2 WT females (Charles River Laboratories, France).

2.1.3. Transfer of the MCH-Ataxin-3 transgene to the C57BL/6 mice

We decided to transfer the transgene to C57BL/6 mice, genetic background widely used in basic sleep research, because FVB/N mice are characterized by a short-onset retinal degeneration. FVB/N MCH^{Atax} males were mated with C57BL/6 WT females (Charles River) until the 8th generation to get a complete transgene transfer (Banbury Conference on genetic background in mice, 1997).

2.1.4. Real-time quantitative PCR analysis

This approach was used to compare expression levels of MCH and orexin mRNA in MCH^{Atax} *vs.* MCH^{WT} after the completion of MCH cell bodies ablation. Adult mice (post-natal weeks 15–20; $n = 16$ for each genotype and genetic background) were sacrificed by cervical dislocation. The hypothalamus (left and right hemispheres) were dissected in sterile ice-cold Ringer's solution and immediately stored at −80 °C. Total RNAs were extracted with the RNeasy Lipid Tissue minikit (Qiagen, Netherlands). Reverse transcription was subsequently performed on 1 μg of total RNAs in a final volume of 20 μL using M-MLV (Invitrogen, USA). cDNAs were amplified by real-time quantitative PCR (LightCycler, Roche, France) using custom primers (Eurofins). Nesfatin-1 and CART mRNAs co-expressed in MCH neurons [5,6] were also quantified in FVB/N mice. Data were normalized to cyclophilin expression using the same sample from the same animal.

Gene	Forward oligonucleotide	Reverse oligonucleotide
MCH	GTATGCTGGGAAGAGTCTAC	ACGTCAAGCATATCGCTTAC
Orexin	CTAGAGCCACATCCCTGCTC	GGGAAGTTTGGATCAGGACA
Nesfatin (Nucb2)	GATGGATTGGACCCTAATGACT	GTTGCTGGTCCAGTGTCTCC
CART	CATCTACGACAAGAAGTACG	CGTCCCTTCACAAGCACTTCA
Cyclophilin	CTGCACTGCCAAGACTGAATG	TTGCCATTCCTGGACCCAAA

2.1.5. Immunohistochemistry

The dual immunodetection of MYC/MCH and MYC/Orexin was done to assess the selective expression of the Ataxin-3 transgene in MCH neurons in MCH^{Atax} mice and to quantify the loss of MCH cell bodies. At post-natal weeks 15–20, MCH^{Atax} and MCH^{WT} mice were deeply anesthetized (sodium pentobarbital, 150 mg/kg, i.p., Ceva Santé Animale, France) and transcardially perfused with Ringer's lactate solution containing 0.1% heparin and 50 mL of an ice-cold fixative (4% paraformaldehyde in 0.1 M PB, pH 7.4). Brains were postfixed for 24 h at 4 °C in the same fixative, dipped in PB containing 30% sucrose for 2 days and finally frozen in cold methyl-butane. Brain free-floating coronal sections (25 μm-thick) were obtained using a cryostat, collected in PBS with 0.3% Triton X100 and submitted to the standard sequential incubations for dual MYC/MCH or MYC/Orexin immunohistochemical staining as previously detailed [5]. Primary antibodies used were biotinylated goat anti-MYC (1:5,000, Vector Laboratories, USA), rabbit anti-MCH (1:50,000, Phoenix Pharmaceuticals, USA), or goat anti-Orexin (1:10,000, Santa Cruz Biotechnology, USA) for 72 h at 4 °C. Secondary biotinylated antisera (1:1,000, 90 min) and ABC Elite kit (1:1,000, 90 min) were purchased from Vector Labs (USA). Double-labeled sections evenly spaced throughout the THA extent (4 sections per mouse) were analyzed with an Axioskop 2 microscope (Zeiss, Germany) equipped with a motorized X–Y-sensitive stage and a video camera connected to a computerized image analysis system (ExploraNova, France). Labeled neurons were bilaterally plotted and automatically counted using Mercator v.2 software (ExploraNova). Photomicrographs were taken with a CCD Color 10-bit QiCam camera, imported into Adobe Photoshop 10 and digitally adjusted for brightness and contrast.

Finally, to determine whether glial cells were activated in the tuberal hypothalamus in MCH^{Atax} *vs.* MCH^{WT} mice at time polysomnographic recordings were done, glial markers were immunodetected using the following primary antibodies: rabbit anti-GFAP (1:10,000, Dako, Denmark); rabbit anti-S100 (1:10,000, Dako, Denmark); rabbit anti-Iba1 (1:5,000, Wako Chemicals, Germany).